The Plant Cell, Vol. 5, 1529–1539, November 1993

An Arabidopsis *myb* Homolog Is Induced by Dehydration Stress and Its Gene Product Binds to the Conserved MYB Recognition Sequence

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An Arabidopsis cDNA (Atmyb2) that contains a sequence that encodes a transcription factor, which is a homolog of MYB, was cloned from a cDNA library prepared from dehydrated Arabidopsis rosette plants. A gene (*Atmyb2*) corresponding to the Atmyb2 cDNA was also cloned and its nucleotide sequence was determined. RNA gel blot analysis showed that the *Atmyb2* mRNA was induced by dehydration and disappeared upon rehydration. The *Atmyb2* mRNA also accumulated upon salt stress and with the onset of treatment with abscisic acid. A β-glucuronidase reporter gene driven by the *Atmyb2* promoter was induced by dehydration and salt stress in transgenic Arabidopsis plants. These observations indicate that *Atmyb2* is responsive to dehydration at the transcriptional level. The putative protein (ATMYB2) encoded by *Atmyb2* has 274 amino acids, a molecular mass of 32 kD, and a putative DNA binding domain that shows considerable homology to plant MYB-related proteins, such as maize C1. A fusion protein that included ATMYB2 was expressed in *Escherichia coli*, and it bound specifically to oligonucleotides that contained a consensus MYB recognition sequence (TAACTG), such as is found in the simian virus 40 enhancer and the maize *bronze-1* promoter. Binding was sequence specific, as indicated by a gel mobility shift experiment. These results suggest that a MYB-related transcription factor is involved in the regulation of genes that are responsive to water stress in Arabidopsis.

INTRODUCTION

In higher plants, a number of genes involved in transcriptional regulation have been cloned and characterized at the molecular level. Among these genes, the *C1* gene from maize was the first to be isolated and extensively characterized. The maize *C1* gene regulates the expression of structural genes that are involved in the biosynthesis of anthocyanin during seed development, and it encodes a putative protein with the conserved DNA binding domain of the c-*myb* proto-oncogene (Paz-Ares et al., 1987). Mammalian MYB proteins have been shown to bind to DNA in a sequence-specific manner (for a review, see Lüscher and Eisenman, 1990). The DNA binding domain of these proteins has three repetitive sequences that contain conserved tryptophan residues in each repeat, and each repeat is known as a "tryptophan cluster" (Kanei-Ishii et al., 1990). The tryptophan residues form a cluster in a hydrophobic core in each repeat and stabilize the structure of the DNA binding domain. However, the DNA binding activity of MYB-related proteins has not been demonstrated in plants. The C termini of MYB proteins each contain a region that functions as a transcriptional activation domain. *C1* also has a C-terminal region that has been shown to be involved in activation of transcription in plants (Goff et al., 1991).

Recently, *myb*-related genes have been reported in maize (Grotewold et al., 1991), barley (Marocco et al., 1989), Antirrhinum (Jackson et al., 1991), petunia (Avila et al., 1993), Arabidopsis (Oppenheimer et al., 1991; Shinozaki et al., 1992), and Physcomitrella (Leech et al., 1993). The barley *myb* homolog, *Hv1*, may be involved in regulation of genes of the anthocyanin biosynthetic pathway, as is the maize *C1* gene. The *myb*-related genes of Antirrhinum, known as *AM340* and *AM306*, may be involved in the regulation of the extracellular secretion of carbohydrate and in the expression of genes that are active in dark-grown tissue, respectively. The Arabidopsis *myb*-related gene *Glabrous1* (*GL1*) is required for the formation of leaf trichomes. DNA gel blot analysis of barley genomic DNA suggests that the barley genome contains many *myb*-related genes (Marocco et al., 1989). Six and seven different cDNAs for *myb* homologs have been reported in Antirrhinum and Arabidopsis, respectively (Jackson et al., 1991; Oppenheimer

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et al., 1991; Shinozaki et al., 1992). By contrast, only one and three genes for *myb* homologs have been cloned from Drosophila and humans, respectively (Katzen et al., 1985; Nomura et al., 1988). Thus, plant *myb*-related genes constitute a rather large family of genes and may play a wide variety of roles in the regulation of gene expression.

Plants respond to drought conditions by exhibiting physiological as well as biochemical changes. Recently, a number of genes have been reported to respond to water deficit at the transcriptional level (Bray, 1988, 1991; Guerrero and Mullet, 1988; Mundy and Chua, 1988; Close et al., 1989; Bartels et al., 1990; Guerrero et al., 1990; Skriver and Mundy, 1990). Most of the drought-inducible genes are also induced by the plant hormone abscisic acid (ABA) (for reviews, see Skriver and Mundy, 1990; Bray, 1991). Many ABA-responsive genes are also expressed at the late stage of seed development (Quatrano, 1987; Baker et al., 1988; Mundy and Chua, 1988; Skriver and Mundy, 1990). The maize *C1* gene is also regulated by ABA during seed development (Hattori et al., 1992).

In the present study, we examined whether a *myb*-related gene is involved in the ABA-responsive expression of drought-inducible genes in vegetative tissues of a higher plant. We screened a cDNA library prepared from Arabidopsis plants that had been dehydrated for 10 hr and cloned a cDNA that encoded a MYB-related protein. We analyzed the structure and expression of the *myb*-related gene, designated *Atmyb2*. The expression of *Atmyb2* was induced by water stress, high-salt conditions, and treatment with ABA at the transcriptional level. We also demonstrated that an ATMYB2 fusion protein expressed in *Escherichia coli* bound to the conserved MYB recognition sequence (PyAACTG) in a sequence-specific manner. We discuss the role of *Atmyb2* in the signal transduction pathway between dehydration stress and gene expression.

RESULTS

Cloning and Sequence Analysis of a *myb*-Related Gene from Arabidopsis That Is Induced by Dehydration

Comparison of the amino acid sequences of reported MYB-related proteins in plants revealed the presence of two highly conserved regions. We designed a set of synthetic oligonucleotide primers that corresponded to these regions and performed polymerase chain reaction (PCR) using these oligonucleotides as primers and cDNAs prepared from stem tissues, flower tissues, and dehydrated Arabidopsis rosette plants. The PCR-amplified products of 180 bp in length, which corresponded to the expected length of the conserved region, were obtained with each template. The PCR products were cloned into the pBluescript II SK− vector and sequenced. Two, four, and one amplified products with sequence homology to the conserved region of MYB were isolated from cDNAs prepared from stem tissues, flower tissues, and dehydrated plants, respectively (data not shown). The deduced amino acid sequences of the seven PCR fragments were not identical to those encoded by four *myb*-related genes, which included *GL1* (Oppenheimer et al., 1991), or to a *myb* homolog (*Atmyb1*) from Arabidopsis (Shinozaki et al., 1992). Therefore, the Arabidopsis genome appears to contain more than 12 *myb*-related genes.

We screened a cDNA library prepared from dehydrated Arabidopsis rosette plants with a PCR-amplified DNA fragment obtained with cDNA prepared from dehydrated plants as a probe. One cDNA clone, Atmyb2, that contained a 1.2-kb insert was isolated. We also screened a genomic library using the cDNA clone as probe and isolated one genomic clone that contained a 4.8-kb DNA fragment. The nucleotide sequences of Atmyb2 cDNA and its corresponding genomic DNA, *Atmyb2*, were determined. The nucleotide and deduced amino acid sequences of *Atmyb2* are shown in Figure 1. Comparison of the genomic sequence with the cDNA sequence revealed that the gene has two introns, a first intron of 91 bp and a second intron of 101 bp. The longest open reading frame extends 819 bp from an ATG initiation codon found at position 982 to a TAA stop codon found at position 1993. A putative TATA box was

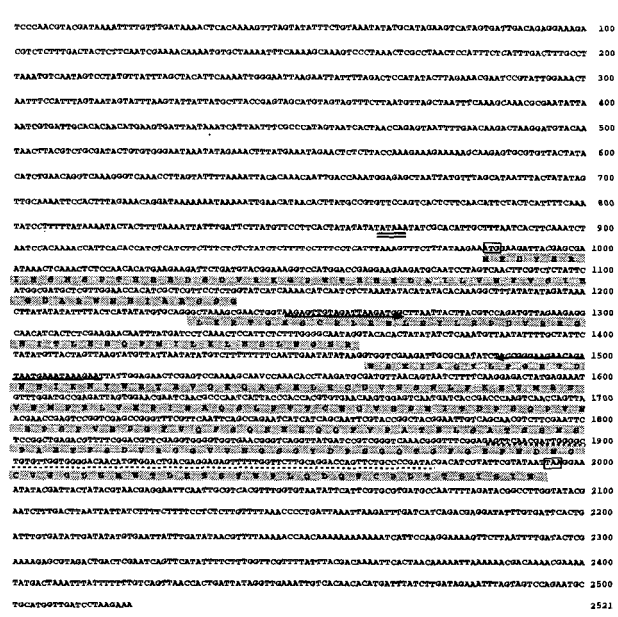

Figure 1. Nucleotide and Deduced Amino Acid Sequences of *Atmyb2*.

The nucleotide sequence of the noncoding strand, including the 5′ and 3′ flanking regions, is shown. The amino acid sequence of the coding region is shown in the single-letter code with shaded boxes below the nucleotide sequence. The putative TATA box is double underlined. The codons for initiation and termination of translation are boxed. The primers for amplification by PCR of the conserved regions of *myb* genes are indicated by arrows. The acidic region in the C-terminal sequence is underlined with a broken line. This nucleotide sequence data has been submitted to DDBJ, EMBL, and GenBank as accession number D14712.

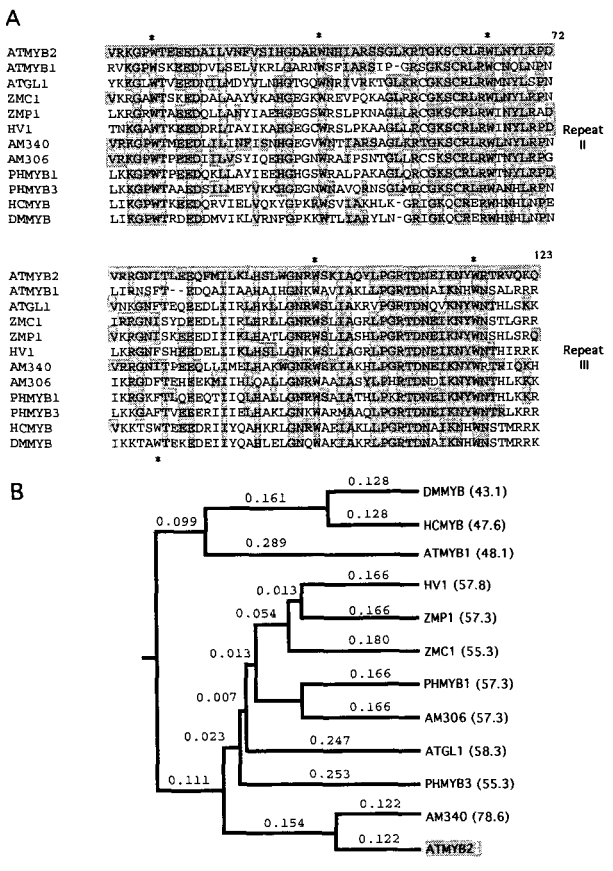

Figure 2. Comparison of Amino Acid Sequences of the DNA Binding Domains of MYB-Related Proteins.

(A) The deduced amino acid sequence of ATMYB2 is compared with the DNA binding domains of MYB-related proteins, namely, maize C1 (ZMC1; Paz-Ares et al., 1987), maize PI (ZMPI; Grotewold et al., 1991), barley Hv1 (HV1; Marocco et al., 1989), Antirrhinum 340 and 306 (AM340 and AM306, respectively; Jackson et al., 1991), petunia MYB1 and 3 (PHMYB1 and PHMYB3, respectively; Avila et al., 1993), Arabidopsis GL1 (ATGL1; Oppenheimer et al., 1991), Arabidopsis ATMYB1 (ATMYB1; Shinozaki et al., 1992), Drosophila MYB (DMMYB; Katzen et al., 1985), and human c-MYB (HCMYB; Majello et al., 1986). Shaded boxes indicate amino acid residues identical to those of ATMYB2, and asterisks represent conserved tryptophan residues. Dashes indicate gaps introduced to maximize alignment. Numbers above each repeat refer to amino acid positions in ATMYB2.

(B) A phylogenetic tree showing evolutional relationship among the MYB proteins was constructed from the matrix of sequence similarities calculated with the UPGMA program (Nei, 1987). Numbers above the horizontal lines indicate the evolutional distance between one protein and another. Amino acid identities (%) of the DNA binding domains of MYB-related proteins with that of ATMYB2 are indicated within parentheses.

found at position 863. No typical polyadenylation signal was found in the 3′ untranslated region.

The putative ATMYB2 protein has 274 amino acids and a molecular mass of 31,532 D. ATMYB2 has a basic region at the N terminus between amino acids 36 and 160 (Figure 1). The N-terminal region contains a region that is highly conserved among MYB-related proteins. Mammalian MYB-related proteins contain a DNA binding domain that consists of three repetitive sequences of 51 to 53 amino acids with three perfectly conserved tryptophan residues in each repeat.

Figure 2A shows a comparison of the amino acid sequences of the DNA binding domains of plant MYB-related proteins, Drosophila MYB, and human c-MYB. ATMYB2, like other plant MYB-related proteins, has two imperfect repeats of 51 to 53 amino acids with conserved tryptophan residues. However, the first tryptophan residue in the second repeat (repeat III) found in animal MYB proteins is replaced by an isoleucine or a phenylalanine residue in plants (Figure 2A). A phylogenetic tree indicating the evolutional distance among the MYB proteins is shown in Figure 2B. The putative DNA binding domain of ATMYB2 shows a higher degree of homology to the plant MYB-related proteins than to human c-MYB. By contrast, ATMYB1 shows higher homology to human c-MYB (59%; Shinozaki et al., 1992) than does ATMYB2 (48%). The presence of several conserved sequences outside the DNA binding domain has been reported in a number of plant MYB proteins (Marocco et al., 1989; Jackson et al., 1991; Avila et al., 1993; Leech et al., 1993). However, we could not find the conserved sequences in the C terminus outside the DNA binding domain of ATMYB2. ATMYB2 contains an acidic region at the C terminus between amino acids 238 and 267 (Figure 2).

Arabidopsis nuclear DNA was digested separately with PstI, XbaI, HindIII, BamHI, and EcoRI and analyzed by DNA gel blotting using the 1.2-kb cDNA clone as a probe. Single HindIII, BamHI, PstI, and XbaI fragments, which had been predicted from the restriction map of *Atmyb2*, hybridized with the cDNA probe under high-stringency conditions (0.1 × SSC, 0.1% SDS at 65°C) (data not shown). The same result was obtained under low-stringency conditions (0.5 × SSC, 0.5% SDS at 55°C) (data not shown). These results suggest that *Atmyb2* does not have sufficient sequence homology to other *myb*-related genes in Arabidopsis to cross-hybridize.

Analysis of the Effects of Dehydration, Exogenous ABA, Salt, Heat, and Cold on the Expression of *Atmyb2*

Atmyb2 was isolated by screening the cDNA library that had been prepared from rosette plants dehydrated for 10 hr. To determine whether *Atmyb2* could be induced by drought stress, we performed RNA gel blot analysis. The *Atmyb2* mRNA appeared within 2 hr, as shown in Figure 3A, of the start of dehydration and its level increased up to 10 hr. Upon rehydration of the dehydrated plants, the level of the transcript decreased within 24 hr and returned to the same level as that in undehydrated plants within 2 days (Figure 3B). These observations indicate that *Atmyb2* is induced by water stress. Many drought-inducible genes have been shown to be responsive to the application of exogenous ABA (Skriver and Mundy,

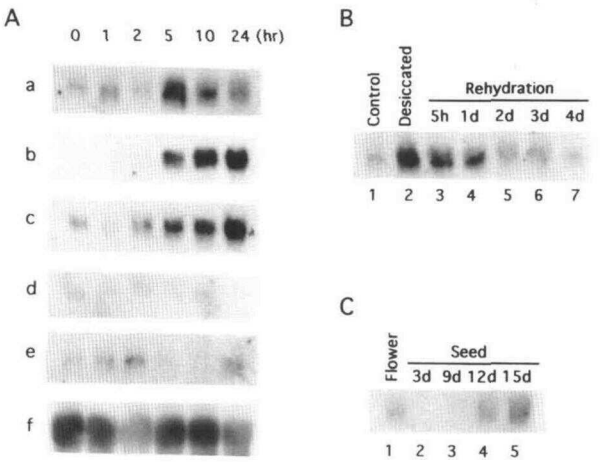

Figure 3. RNA Gel Blot Analysis of Expression of the *Atmyb2* Gene.

(A) Induction of *Atmyb2* by dehydration stress, high-salt conditions, application of exogenous ABA, and heat and cold. Each lane was loaded with 30 μg of total RNA prepared from unbolted Arabidopsis plants that had been dehydrated (a), transferred from agar plates to hydroponic growth in 250 mM NaCl (b) or in 100 μM ABA (c), or transferred to and grown at 40°C (d) or 4°C (e). Numbers above each lane indicate the time in hours (hr) after the initiation of treatment before isolation of RNA. A control RNA gel blotting of dehydrated plants with 18S rRNA as a probe is also shown (f).

(B) Expression of *Atmyb2* after reversal of dehydration. Each lane contains 30 μg of total RNA prepared from rosette plants that had been dehydrated for 5 hr (lane 2) or dehydrated for 5 hr and then rehydrated in GM solution (Valvekens et al., 1988) for the indicated periods of time (hours, h, and days, d; lanes 3 to 7). RNA in lane 1 was isolated from control plants that had been exposed only to standard growth conditions.

(C) Expression of *Atmyb2* during maturation of seeds. Each lane contains 30 μg of total RNA prepared from flowers (lane 1) or developing seeds (including siliques) that had been harvested at the indicated number of days after anthesis (lanes 2 to 5).

1990; Bray, 1991). Therefore, we examined the effect of treatment with exogenous ABA on the expression of *Atmyb2* by RNA gel blot analysis. The expression of *Atmyb2* was found to be induced by exogenous ABA (Figure 3A), but not by a control treatment with water alone (data not shown). To examine the effect of salt stress on the expression of *Atmyb2*, we performed RNA gel blotting with total RNA isolated from whole rosette plants that had been treated with 250 mM NaCl for various periods. Figure 3A shows that *Atmyb2* was induced under high-salt conditions. These results suggest that a change in the osmotic potential of the environment can serve as a trigger for the induction of *Atmyb2*. We then examined the effects of other environmental stresses, such as cold and heat, on the expression of *Atmyb2*. Arabidopsis plants grown at 22°C were exposed to temperatures of 4 or 40°C. No *Atmyb2* mRNA accumulated under these conditions (Figure 3A).

The level of ABA in seeds increased at the middle stage of seed development. The maize *C1* gene is specifically expressed at the late stage of embryogenesis and is induced by ABA (Hattori et al., 1992). Therefore, we examined the level of *Atmyb2* mRNA during seed maturation by RNA gel blot analysis. The *Atmyb2* mRNA appeared at the late stage of seed development (12 days after anthesis) and accumulated in mature seeds (15 days; Figure 3C). Thus, *Atmyb2* is induced during seed maturation, as well as by dehydration or salt stress in vegetative tissues.

A β-Glucuronidase Reporter Gene Driven by the *Atmyb2* Promoter Is Induced by Desiccation Stress and Salt Stress at the Transcriptional Level

We analyzed the 2-kb 5′ flanking sequence of *Atmyb2* for its involvement in drought-inducible expression in transgenic Arabidopsis. We constructed a chimeric gene, shown in Figure 4A, that consisted of the *Atmyb2* promoter fused to a β-glucuronidase (*GUS*) reporter gene (*Atmyb2*p–*GUS*). The *Atmyb2* promoter included the sequence from 2 kb upstream to 100 bp downstream of the site of initiation of translation. Arabidopsis plants were transformed with the *Atmyb2*p–*GUS* fusion gene. RNA gel blot analysis revealed that the level of the *GUS* mRNA under control of the *Atmyb2* promoter, as well as that of endogenous *Atmyb2* mRNA, was clearly increased by dehydration (Figure 4B) and high-salt conditions (data not shown). However, the maximum level of induction of the *GUS*

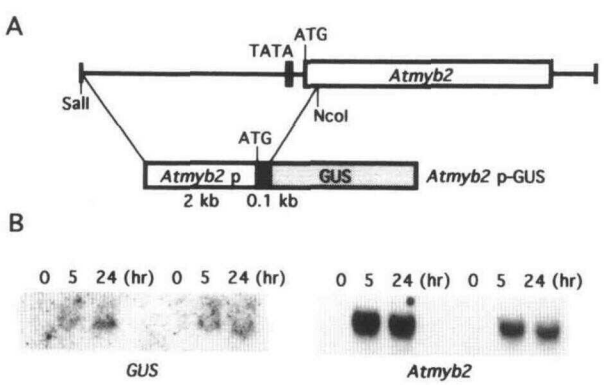

Figure 4. Induction of the *Atmyb2*p–*GUS* Fusion Gene in Transgenic Arabidopsis Plants after Exposure to Dehydration or Salt Stress.

(A) A construct used in transgenic plant experiments. A SalI-NcoI fragment containing 2 kb of the region upstream and 100 bp of the region downstream from the site of initiation of translation (ATG) of the *Atmyb2* gene was fused to the coding region of a *GUS* reporter gene as described in Methods.

(B) RNA gel blot analysis of the induction of *GUS* and *Atmyb2* mRNAs in transgenic Arabidopsis plants after exposure to dehydration. Each lane contains 30 μg of total RNA prepared from two independent lines that had been dehydrated for the indicated periods of time. Hybridization was performed using the Atmyb2 cDNA and the GUS cDNA as probes.

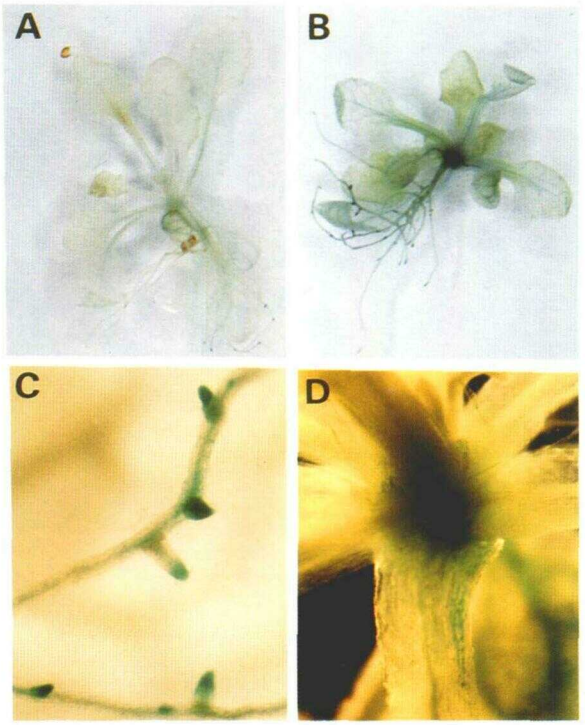

Figure 5. Histochemical Localization of GUS Activity in Transgenic Arabidopsis Plants.

(A) Plant grown under normal conditions.
(B) to **(D)** Plants exposed to high-salt conditions. Strong GUS activity was observed in root tips **(C)** and petioles and leaf bases **(D)** from transgenic Arabidopsis plants that had been transferred from agar plates to 250 mM NaCl and allowed to grow hydroponically for 12 hr.

mRNA was lower than that of the *Atmyb2* mRNA under water deficit conditions. These results indicate that the drought-inducible expression of *Atmyb2* is mainly regulated at the transcriptional level and that the 2-kb promoter region contains a *cis* element(s) that is involved in desiccation-responsive expression. The low level of the dehydration-induced *GUS* mRNA may be due to the instability of the fused *GUS* mRNA or the lack of enhancer-like sequences in the used DNA fragment of the *Atmyb2* promoter.

We analyzed the tissue-specific expression of the GUS activity that was driven by the *Atmyb2* promoter in transgenic Arabidopsis plants exposed to high-salt conditions, as shown in Figure 5. GUS activity was clearly detected in root tips and leaf bases, and weak GUS activity was observed in petioles of transgenic Arabidopsis rosette plants that had been exposed to salt stress as well as in those that had been exposed to dehydration stress (data not shown). Weak GUS expression was observed in lateral root tips of the untreated control plants, which may reflect the tissue-specific expression of *Atmyb2* under normal growth conditions.

Analysis of the DNA Binding Activity of the ATMYB2 Fusion Protein Expressed in *Escherichia coli*

To examine whether the ATMYB2 and ATMYB1 proteins have DNA binding activity, we performed a gel mobility shift assay with the recombinant ATMYB2 and ATMYB1 proteins that had been expressed in *E. coli* and an oligonucleotide probe that contained the c-MYB binding sequence found in the simian virus 40 (SV40) enhancer region (Nakagoshi et al., 1990). The c-MYB binding sequence contains a TAACTG sequence in the middle. We also analyzed the DNA binding activity of the recombinant murine c-MYB protein (Ramsay et al., 1989) as a control. Figure 6A shows the construction of fusion genes

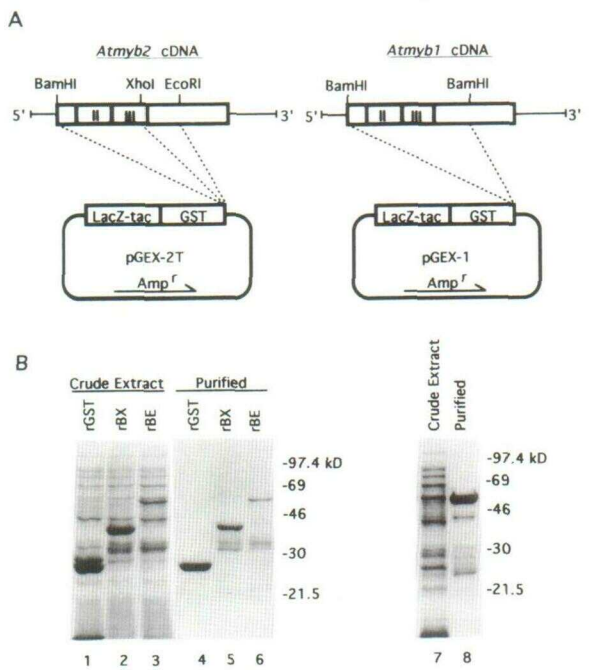

Figure 6. Expression of ATMYB1 and ATMYB2 Fusion Proteins in *E. coli*.

(A) Construction of pGEX-rATMYB2BE, pGEX-rATMYB2BX, and pGEX-rATMYB1 for expression of ATMYB2 and ATMYB1 proteins. A 620-bp BamHI-EcoRI fragment (rATMYB2BE) and a 350-bp BamHI-XhoI fragment (rATMYB2BX) derived from the Atmyb2 cDNA were introduced into pGEX-2T (left). A 710-bp BamHI fragment (rATMYB1) derived from the Atmyb1 cDNA was introduced into pGEX-1 (right).
(B) SDS-PAGE analysis of the proteins expressed in *E. coli* cells that carried pGEX-rATMYB2BE, pGEX-rATMYB2BX, or pGEX-rATMYB1. Crude extracts prepared from isopropyl β-D-thiogalactopyranoside–treated *E. coli* cells that carried the pGEX vector (rGST; lane 1), pGEX-rATMYB2BX (rBX; lane 2), pGEX-rATMYB2BE (rBE; lane 3), and pGEX-rATMYB1 (lane 7) were analyzed by SDS-PAGE. Fusion proteins purified with glutathione-Sepharose, which had been prepared from *E. coli* cells that carried the pGEX vector (rGST; lane 4), pGEX-rATMYB2BX (rBX; lane 5), pGEX-rATMYB2BE (rBE; lane 6), and pGEX-rATMYB1 (lane 8), were also analyzed by SDS-PAGE.

with the Atmyb2 and Atmyb1 cDNAs cloned in the multicloning site of the glutathione *S*-transferase (GST) expression vector, pGEX (Smith and Johnson, 1988). Since it has been shown that C-terminal truncation of the mouse c-MYB protein has no effect on binding to DNA (Howe et al., 1990), we constructed two fusion genes as follows.

The first consisted of the gene encoding GST and a 620-bp BamHI-EcoRI fragment of Atmyb2 cDNA, which encodes a protein that lacks 66 of the amino acid residues at the C terminus of the parent protein (the plasmid was designated pGEX-rATMYB2BE). The second fusion gene consisted of the gene encoding GST and a 350-bp BamHI-XhoI fragment of Atmyb2 cDNA that encodes a protein that lacks most of the C terminus and five amino acid residues of repeat III of the DNA binding domain (pGEX-rATMYB2BX). We also constructed a fusion construct with the gene encoding GST and a 710-bp BamHI fragment of the Atmyb1 cDNA that encodes a protein which lacks 154 amino acid residues of the C terminus (pGEX-rATMYB1).

E. coli cells carrying these recombinant plasmids were grown, and the production of recombinant proteins was induced by the addition of isopropyl β-D-thiogalactopyranoside. The fusion proteins were partially recovered in the soluble fractions of crude extracts prepared from *E. coli* cells that had been grown at 25°C, and the fusion proteins were purified from the crude extracts by affinity chromatography on glutathione-Sepharose (Figure 6B). Several extra products in addition to predicted bands of protein were observed and were assumed to be premature versions or products of degradation of the fusion proteins, because no extra bands were observed in the analysis of the purified protein fraction prepared from cells that carried the pGEX vector alone (Figure 6B, lane 4).

The purified fusion proteins (rATMYB2BE, rATMYB2BX, and rATMYB1) were examined for their ability to bind to an oligonucleotide that contained the MYB binding sequence found in the SV40 enhancer (MBSI, MYB-binding site I; Nakagoshi et al., 1990) in the gel mobility shift assay. To analyze the specificity of the DNA binding activities, we used a mutated form of MBSI (mMBSI) as well as MBSI as probes, as shown in Figure 7A. The mMBSI oligonucleotide contained two point mutations in the MYB binding site (TCCCTG instead of TAACTG; Figure 7A). rATMYB2BE as well as rATMYB1 and c-MYB proteins bound to MBSI, but not to mMBSI, as shown in Figure 7A, lanes 3, 5, and 6. By contrast, rGST (GST protein expressed in *E. coli* cells with the pGEX vector) and rATMYB2BX did not bind to MBSI or mMBSI (Figure 7A, lanes 2 and 4). The inability of rATMYB2BX to bind to MBSI suggests that the second repeat (repeat III) of the DNA binding domain is necessary for the sequence-specific binding. This result is consistent with a previous observation made with mouse c-MYB (Howe et al., 1990). In the gel mobility shift assay, the DNA binding activity of rATMYB2BE and rATMYB1 to MBSI was reduced by the addition of excess unlabeled MBSI, but not by that of unlabeled mMBSI, as shown in Figure 8A. These results indicate that both rATMYB2BE and rATMYB1 bind sequence specifically to the MBSI oligonucleotide.

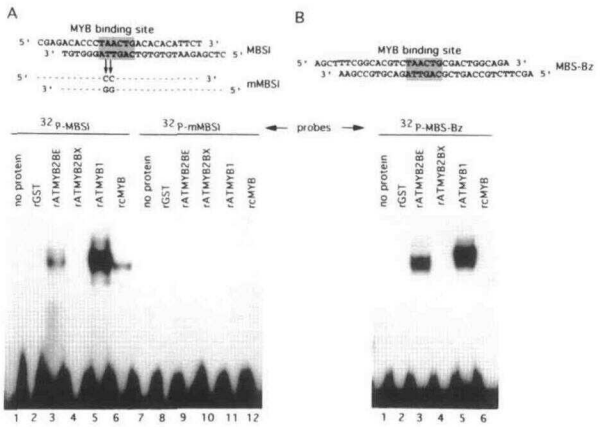

Figure 7. DNA Binding Properties of the ATMYB2 and ATMYB1 Fusion Proteins That Had Been Expressed in *E. coli.*

(A) Sequence-specific binding of the recombinant proteins to MBSI, which is found in the SV40 enhancer. MBSI contains a consensus MYB binding site (TAACTG), indicated by a shaded box, and mMBSI contains two point mutations (TCCCTG) in the MYB binding site (Nakagoshi et al., 1990). DNA binding reactions were performed with 2 μg of rATMYB2BE (lanes 3 and 9), 2 μg of rATMYB2BX (lanes 4 and 10), 0.2 μg of rATMYB1 (lanes 5 and 11), 0.2 μg of rc-MYB (lanes 6 and 12), or 1 μg of rGST (lanes 2 and 8), and ^{32}P-labeled MBSI (lanes 1 to 6) or mMBSI (lanes 7 to 12).

(B) Sequence-specific binding of the recombinant proteins to MBS-Bz, which is found in the *Bz-1* promoter. The MBS-Bz oligonucleotide is derived from the *Bz-1* promoter and contains a consensus MYB binding site that is indicated by a shaded box (Roth et al., 1991). DNA binding reactions were performed with 0.1 μg of rATMYB2BE (lane 3), 2 μg of rATMYB2BX (lane 4), 0.2 μg of rATMYB1 (lane 5), 0.6 μg of rc-MYB (lane 6), or 1 μg of rGST (lane 2) and ^{32}P-labeled MBS-Bz (lanes 1 to 6).

The product of the maize *C1* gene regulates the transcription of five genes, *A1*, *A2*, *Bronze-1* (*Bz-1*), *Bz-2*, and *C2*, that encode enzymes involved in an anthocyanin biosynthetic pathway (Coe et al., 1988). The 5′ flanking region of *Bz-1*, containing the MYB consensus binding sequence, has been shown to be essential for the expression of *Bz-1* (Roth et al., 1991). Overexpression of *C1* in aleurone tissue results in *trans*-activation of the *Bz-1* promoter (Goff et al., 1991). To examine whether rATMYB2BE binds to the MYB recognition sequence in the *Bz-1* promoter, we performed a gel mobility shift assay with an oligonucleotide that contained a MYB binding sequence in the *Bz-1* promoter (MBS-Bz) as probe (Figure 7B). rATMYB2BE as well as rATMYB1 and c-MYB bound to MBS-Bz, which suggests that MBS-Bz functions as a target sequence for MYB DNA binding proteins (Figure 7B, lanes 3, 5, and 6). We then performed a gel mobility shift assay with MBSI as probe and MBS-Bz as competitor to analyze the binding affinity of ATMYB2 and ATMYB1 proteins to MBSI and MBS-Bz. We used the same amount of recombinant proteins in each binding assay. The extent of binding of rATMYB2BE to MBSI was reduced more effectively by the addition of

unlabeled MBS-Bz (Figure 8A, lanes 7 to 9) than by the addition of unlabeled MBSI (Figure 8A, lanes 1 to 3). By contrast, the competition by MBS-Bz was not as effective in the case of rATMYB1 (Figure 8B, lanes 7 to 9), but MBSI reduced the extent of binding of rATMYB1 to MBSI more effectively than MBS-Bz did. Mutant mMBSI had no effect on the extent of binding of the two recombinant proteins to MBSI (Figures 8A and 8B, lanes 4 to 6). These observations suggest that rATMYB2BE binds preferentially to MBS-Bz rather than to MBSI, whereas rATMYB1 binds preferentially to MBSI rather than to MBS-Bz.

DISCUSSION

We isolated a gene that encodes a homolog of the transcription factor MYB (*Atmyb2*) from Arabidopsis. The gene is induced by drought stress and high-salt conditions. RNA gel blot analysis showed that *Atmyb2* mRNA accumulated upon dehydration and high-salt treatment but not as a result of cold or heat stress (Figure 3A). Rehydration of dehydrated Arabidopsis plants caused a decrease in the level of *Atmyb2* mRNA, a result that indicates that *Atmyb2* responds to water stress (Figure 3B). We analyzed the 5′ flanking sequence of *Atmyb2* by transforming Arabidopsis with a *GUS* fusion construct. GUS activity driven by the *Atmyb2* promoter was induced by high-salt conditions as well as by dehydration (Figure 4B). These results indicate that the induction of *Atmyb2* by dehydration or salt stress occurs at the transcriptional level and is not due to the induction of increased stability of the corresponding mRNA. However, the extent of induction was lower than that of *Atmyb2* mRNA, perhaps because of instability of the mRNA transcribed from the fusion gene or lack of an enhancer sequence in the 2-kb upstream region.

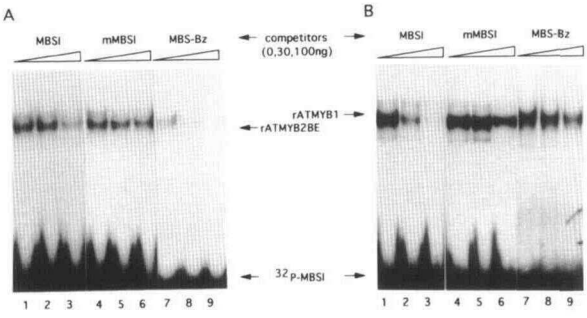

Figure 8. Characterization of DNA Binding Affinities of rATMYB2BE and rATMYB1 to MBSI and MBS-Bz Oligonucleotides.

(A) Competitive DNA binding assay of rATMYB2BE. The DNA binding reaction was performed by preincubating unlabeled competitor MBSI (lanes 1 to 3), mMBSI (lanes 4 to 6), and MBS-Bz (lanes 7 to 9) with 2 μg of rATMYB2BE with the subsequent addition of ^{32}P-labeled MBSI. Amounts of competitor (nanograms) added to reaction mixtures are indicated.

(B) Competitive DNA binding assay of rATMYB1. The DNA binding reaction was performed by preincubating unlabeled competitor MBSI (lanes 1 to 3), mMBSI (lanes 4 to 6), and MBS-Bz (lanes 7 to 9) with 0.2 μg of rATMYB1 with the subsequent addition of ^{32}P-labeled MBSI. Amounts of competitor (nanograms) added to reaction mixtures are indicated.

Atmyb2 was also induced by the application of exogenous ABA (Figure 3A). The biosynthesis of ABA is induced by water deficiency, and the increased level of ABA results in the induction of various ABA-responsive genes. Therefore, many genes that respond to dehydration stress are also induced by exogenous ABA. A highly conserved sequence, namely, PyACGTGG, has been found in the 5′ upstream regions of many ABA-responsive genes, and it is thought to function in the ABA-responsive transcription of wheat *Em* and rice *rab16* genes (Marcotte et al., 1989; Mundy et al., 1990). However, the conserved sequence known as ABRE (ABA-responsive element) was not found in the 5′ upstream sequence of *Atmyb2.* The maize *C1* gene is also induced by ABA, and it is regulated by *Viviparous-1* (*Vp1*), which encodes a transcription activator that is not a DNA binding protein (McCarty et al., 1991). A *cis*-acting element involved in ABA-responsive and *Vp1*-regulated gene expression of *C1* was identified as GGTCGTGTCGTCCATGCATGCAC (the underlined sequence is called a SphI element) by a cotransfection assay with maize protoplasts (Hattori et al., 1992). A 21mer oligonucleotide repeated four times and containing the hex-3 sequence (TTCGGCCACGCGTCCAATCCG), a mutant version of the hex-1 sequence (with ACGTCA changed to ACGCGT), was demonstrated to function as an ABA-responsive element in transgenic tobacco (Lam and Chua, 1991). We failed to find these sequences in the 5′ flanking region of *Atmyb2* (Figure 2). Therefore, the *cis*-acting element(s) involved in the ABA-responsive expression of *Atmyb2* is likely to be different from those of previously reported ABA-responsive genes.

The putative ATMYB2 protein has several features common to plant homologs of MYB. The ATMYB2 protein as well as other plant MYB proteins lack repeat I in the DNA binding domain that has been found in human, mouse, and Drosophila MYB proteins (Figure 2A). Products of viral *myb* oncogenes (v-*myb*) also interact with DNA in a sequence-specific manner in spite of the absence of repeat I (Biedenkapp et al., 1988). In the case of mouse c-MYB, deletion of repeat I has no effect on the DNA binding activity (Howe et al., 1990). The absence of five amino acid residues from the second repeat, which corresponds to the repeat III, caused the loss of the DNA binding activity of rATMYB2 expressed in *E. coli* (Figure 7A). These findings imply that only repeats II and III are required for a functional DNA binding domain in plant MYB proteins.

The c-MYB protein recognizes the conserved DNA sequence PyAACTG, and AAC has been proposed as the core sequence of the binding site (Biedenkapp et al., 1988; Nakagoshi et al., 1990). The rATMYB2BE, rATMYB1, and c-MYB proteins that are expressed in *E. coli* were shown to bind to two oligonucleotides that contained the conserved MYB recognition sequence (MRS). They were MBSI, which contained the MRS found in the SV40 enhancer, and MBS-Bz, which contained the MRS

found in the maize *Bz-1* promoter. However, the binding affinities of rATMYB2BE and rATMYB1 for MBSI and MBS-Bz seemed to be different. The binding affinity of rATMYB2BE for MBS-Bz was slightly higher than that for MBSI (Figure 8A). By contrast, the binding affinity of rATMYB1 for MBS-Bz was lower than that for MBSI (Figure 8B). Sequence analysis indicates that ATMYB2 is more similar to the product of the plant *myb* genes, whereas ATMYB1 is highly homologous to human c-MYB (Figure 2B). The difference between the amino acid sequences of these MYB proteins seems likely to be responsible for the differences in DNA binding affinities for target sequences. Because both MBSI and MBS-Bz contain the same consensus sequence TAACTG, differences in the flanking sequences may affect the DNA binding affinities of the MYB proteins.

Because *Atmyb2* is induced by water stress, it seems likely that its gene product functions as a transcription factor that controls the expression of genes induced by drought stress or high-salt conditions. We have isolated nine cDNAs whose corresponding genes (*rd*) are induced by dehydration stress, and we have analyzed their expression (Yamaguchi-Shinozaki et al., 1992). Two of the drought-responsive genes in Arabidopsis, *rd*22 and *rd*29B, show a pattern of gene expression similar to that of *Atmyb2* (Yamaguchi-Shinozaki and Shinozaki, 1993). *rd*22, *rd*29B, and *Atmyb2* are induced by dehydration and salt stress but not by cold and heat stress. ABA causes the induction of both *rd*22 and *Atmyb2*. Moreover, protein synthesis is necessary for the induction of *rd*22 and *rd*29B (Yamaguchi-Shinozaki and Shinozaki, 1993). The 5′ flanking region of *rd*22 contains two MYB recognition sequences to which ATMYB2 was able to bind, as demonstrated by a gel mobility shift assay (T. Urao and K. Shinozaki, unpublished observation). The *rd*29B promoter also contains a MYB recognition sequence. These observations raise the possibility that the product of the *Atmyb2* gene functions as a transcription factor in the expression of *rd*22 and *rd*29B. ABA-induced accumulation of proteinase inhibitor II mRNA also required protein synthesis (Peña-Cortés et al., 1989), which indicates that factors mediating the induction of PI-II are newly synthesized upon ABA treatment. The ATMYB2 homolog may be one such factor in PI-II induction by ABA.

The ATMYB2 protein expressed in *E. coli* was shown to bind to the MYB recognition sequence of the maize *Bz-1* promoter (Figure 7B). Recently, maize C1 and B proteins have been demonstrated to interact directly on the *Bz-1* promoter and to *trans*-activate cooperatively the *Bz-1* promoter (Goff et al., 1992). Genetic analysis indicates that at least five genes encoding enzymes involved in the biosynthesis of anthocyanin, namely *Bz-1*, *A1*, *C2*, *Bz-2*, and *A2*, are regulated by the *C1* family (*C1*, *Pl*, and *Bh*) and *R* family (*R*, *B*, and *Sn*) of genes (Coe et al., 1988). Proteins in the *R* family contain regions homologous to the basic helix-loop-helix DNA binding/dimerization domain found in MYC DNA binding proteins (Ludwig et al., 1989; Perrot and Cone, 1989; Radicella et al., 1991). Cooperative activation of MYB proteins with other transcription factors has also been reported in animal and yeast. The chicken *mim-1* gene that has been identified as a target gene for v-MYB is activated synergistically by v-MYB and the C/EBP transcription factor (Burk et al., 1993). BAS1 that has a DNA binding domain similar to MYB proteins activates transcription of the *HIS4* gene only in combination with BAS2, which contains a homeobox in yeast (Tice-Baldwin et al., 1989). ATMYB2 may also require a second protein(s), such as the MYC homologs, for modulation of its DNA binding properties and/or efficient activation of target genes because two MYC recognition sequences are located close to the MYB recognition sequence in the dehydration-responsive *rd*22 promoter (Yamaguchi-Shinozaki and Shinozaki, 1993).

METHODS

Plant Growth and Stress Treatments

Arabidopsis (Columbia ecotype) plants were grown on GM agar plates (Valvekens et al., 1988) under continuous illumination of ~2500 lux at 22°C for 4 to 5 weeks and used in stress treatment experiments prior to bolting. Arabidopsis rosette plants were harvested from GM agar plates and then dehydrated on Whatman No. 3MM paper (Whatman International, Maidstone, England) at 22°C and 60% humidity under dim light. Plants subjected to treatment with abscisic acid (ABA) and to salt stress were grown hydroponically in a solution of 100 μM ABA and 250 mM NaCl, respectively, under dim light. Heat and cold treatments were performed under continuous light by exposure of plants grown at 22°C to a temperature of 40 and 4°C, respectively. In each case, the plants were subjected to the stress treatments for various time periods, frozen in liquid nitrogen, and stored at −80°C.

Preparation of a cDNA Library and DNA Templates for the Polymerase Chain Reaction

Total RNA was isolated from stems, flowers, and dehydrated intact rosette plants by the method of Nagy et al. (1988) and purified by chromatography on oligo(dT) cellulose as described elsewhere (Maniatis et al., 1982). Double-stranded cDNA was synthesized from poly(A)$^+$ RNA by use of cDNA synthesis System Plus (Amersham International). cDNA libraries were constructed using cDNA cloning system λgt11 (Amersham). DNA templates for amplification by polymerase chain reaction (PCR) were prepared from the cDNA libraries by phenol extraction and CsCl gradient centrifugation and amplified by PCR using primers that corresponded to the two adapters of the insert cDNAs (Mizoguchi et al., 1993).

Polymerase Chain Reaction

Two oligonucleotide primers corresponding to highly conserved regions of MYB-related proteins were synthesized as follows: forward, 5′-GGI-AA(A/G)TCITG(T/C)(C/A)GI(T/C)TI(C/A)GITGG-3′; reverse, 5′-TTC-CA(G/A)TA(G/A)TTIAT(C/T)IG(C/A)(T/C)GTICT(A/G)AAIGG-3′. The oligonucleotide primers were phosphorylated with T4 polynucleotide kinase as described elsewhere (Maniatis et al., 1982). PCR was performed using a GeneAmp kit according to the manufacturer's

instructions (Perkin-Elmer Cetus, Norwalk, CT). Thirty-five cycles of denaturation, annealing, and polymerization were conducted at 94°C for 1 min, at 43°C for 2 min, and at 72°C for 5 min, respectively. The amplified DNA fragments were purified on a 5% polyacrylamide gel and cloned into the SmaI site of pBluescript II SK− (Stratagene).

Cloning and DNA Sequencing

An Arabidopsis cDNA library prepared from dehydrated rosette plants (as described above) and a genomic DNA library (Clonetech, Palo Alto, CA) were screened by plaque hybridization as described by Maniatis et al. (1982). The probes were labeled with ^{32}P-dCTP using a random primer kit according to the manufacturer's instruction (Boehringer Mannheim). Positive plaques were purified, and the DNA inserts were isolated by phenol extraction and CsCl gradient centrifugation (Maniatis et al., 1982). The cloned DNA fragments were subcloned into pBluescript II SK−. DNA sequences were determined by the dye-primer cycle sequencing method using a DNA sequencer (model 373A; Applied Biosystems, Foster City, CA). The GENETYX (Software Development, Tokyo) and Gene Works (IntelliGenetics, Inc., Mountain View, CA) software systems were used for the analysis of DNA and amino acid sequences.

DNA and RNA Gel Blot Analysis

Thirty micrograms of total RNA was fractionated on a 1% agarose gel that contained formaldehyde and blotted onto a nitrocellulose filter (Maniatis et al., 1982). The filter was hybridized with ^{32}P-labeled cDNA in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 25 mM of sodium phosphate buffer, pH 6.5, 10 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), and 250 μg/mL of denatured salmon sperm DNA at 42°C. The filter was washed twice with 0.1 × SSC, 0.1% SDS at 60°C for 15 min and subjected to autoradiography. Genomic DNA gel blot analysis was performed as described elsewhere (Maniatis et al., 1982).

Transgenic Plants

A 2.1-kb SalI-NcoI fragment containing 2 kb of the region upstream of the site of initiation of translation and 100 bp of the coding region of the *Atmyb2* gene was filled in by treatment with the Klenow fragment of DNA polymerase I and deoxynucleotide triphosphates, and then it was subcloned into the SmaI site of a promoterless β-glucuronidase (*GUS*) expression vector, pBI101.1 (Clonetech). The resulting translational fusion construct, *Atmyb2p–GUS*, was transferred from *Escherichia coli* DH5α into *Agrobacterium tumefaciens* C58C1Rif by triparental mating with *E. coli* that contained plasmid pRK2013. Arabidopsis (Columbia ecotype) plants were transformed with Agrobacterium that contained *Atmyb2p–GUS* by root infection as described elsewhere (Valvekens et al., 1988). Histochemical localization of GUS activities in the transgenic plants was performed by incubating the transgenic unbolted plants in X-gluc buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 2% DMSO, 0.5 mM potassium ferrocyanide, 2 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide [X-gluc]) at 37°C for 6 to 12 hr. The stained plants were fixed in 5% formaldehyde, 5% acetic acid, and 20% ethanol, and then they were washed with 50 to 100% ethanol to remove chlorophyll.

Production and Purification of Glutathione S-Transferase Fusion Proteins

A 620-bp BamHI-EcoRI fragment, a 350-bp BamHI-XhoI fragment derived from Atmyb2 cDNA, and a 710-bp BamHI fragment derived from Atmyb1 cDNA were cloned into the pGEX vector (Smith and Johnson, 1988), and then recombinant plasmids were used to transform in *E. coli* JM109 cells. *E. coli* cells containing chimeric constructs were grown in 200 mL of 2 × YT medium (Maniatis et al., 1982) that contained 100 μg/mL ampicillin at 37°C. When absorbance of each culture reached a 0.8 at 600 nm, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the cells were incubated for 10 hr at 25°C. The cells were harvested, washed, and resuspended in 13 mL of lysis buffer (10 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 5 mM $MgCl_2$, 5% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride). Lysozyme was added to a final concentration of 1 mg/mL, the cells were placed on ice for 1 hr, and cells were frozen and thawed twice. Triton X-100 and EDTA were added to final concentrations of 1% (v/v) and 1 mM, respectively.

The cells were sonicated five times for 15 sec each on ice and centrifuged at 22,000*g* for 20 min. The supernatant was mixed with 3 mL of glutathione-Sepharose (Pharmacia, Uppsala, Sweden) and rocked for 4 hr at 4°C. The Sepharose beads were washed five times with lysis buffer, and the fusion protein was eluted from the beads by incubation for 30 min with 50 mM Tris-HCl, pH 9.6, that contained 5 mM of reduced glutathione. The eluant was dialyzed against 50 mM Tris-HCl, pH 7.0, and stored at −80°C. Protein concentrations were determined with a protein assay kit (Bio-Rad).

Gel Mobility Shift Assay

Oligonucleotide probes were labeled by filling in 5′ overhangs with ^{32}P-dCTP and the Klenow fragment. The DNA binding reaction was allowed to proceed for 20 min at 25°C in 20 μL of binding buffer (25 mM Hepes/KOH, pH 7.9, 50 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 5% glycerol, 5 μg/μL BSA) that contained 20,000 dpm of ^{32}P-labeled oligonucleotide probe, 2 μg of poly(dI-dC), and bacterially produced fusion protein that had been purified with glutathione-Sepharose. Competition experiments were performed by adding unlabeled competitor oligonucleotide to the binding reaction with the subsequent addition of radiolabeled oligonucleotide. The reaction mixture was subjected to electrophoresis on a 6% polyacrylamide gel in 0.25 × Tris-borate-EDTA buffer at 100 V for 2 hr. The gel was dried and subjected to autoradiography.

ACKNOWLEDGMENTS

We thank Drs. Shunsuke Ishii, Teruaki Nomura, and Tatsuhiko Sudo of RIKEN for providing us with recombinant c-MYB protein and for helpful discussions. This work was supported in part by the Special Coordination Fund of the Science and Technology Agency of the Japanese Government and a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan to K.S. K.Y.-S. was supported by a fellowship from the Science and Technology Agency of Japan.

Received June 1, 1993; accepted August 20, 1993.

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